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Use Against Breast Cancer in Humans

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13. ABSTRACT (Maximum 200) During the first two years of this project, we established that nanogram quantities of ligand-activated and microgram quantities of unactivated natural and recombinant human AFP inhibited growth of estrogen-dependent MCF-7 human breast cancer. During year three of this project we have established that other estrogen-dependent human breast cancers as well as androgen-dependent human prostate cancer are growth-inhibited by microgram doses of unactivated natural human AFP. Hormone-independent human breast, ovarian, uterine and prostate cancers were not growth-inhibited by AFP. The histopathology of tumors growth-inhibited by AFP continues to be a profile of cytostasis with an accumulation of cells in G ₀ G ₁ and no evidence of necrosis. Receptor for sex steroid hormone appears to be a marker for tumor sensitivity to AFP. Elevation in serum FSH and E ₂ levels are intermediate markers for <i>in vivo</i> effect of AFP against estrogen-dependent responses. Truncated forms of AFP have activity similar to that of the full-length molecule. There has been no evidence of host toxicity during therapeutic application of the active forms of AFP.				
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FOREWORD

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James A. Bennett 6-26-97

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INTRODUCTION (Taken from Year 2 progress report)

Subject: We are studying the regulation of breast cancer growth by alpha-fetoprotein (AFP). AFP is a glycoprotein normally produced during gestation, initially by the fetal yolk sac and then by the fetal liver (1). It is a major protein constituent of the fetal plasma throughout gestation and has structural similarities to albumin (2). However, upon parturition, the gene for AFP is repressed, and its serum concentration diminishes to a negligible level. It is reexpressed during liver pathology such as hepatoma or cirrhosis. The restricted presence of this embryonic protein suggests a unique role for AFP in cell growth and differentiation, which are the hallmarks of embryonic life. Evidence for this role has been obtained in a variety of studies showing that AFP can regulate the growth and function of certain tissues such as liver (3), lymphocytes (4), placenta (5), ovaries (6), and uterus (7), and interact with certain ligands such as arachidonic acid (8), docosahexaenoic acid (8) and retinoic acid (8), all of which influence differentiation. Our own studies have shown that when either rodent or human AFP is incubated with a molar excess of estradiol, the protein undergoes a change in conformation (9). In this transformed state (tAFP), nanogram quantities of the material inhibit the growth of estrogen-stimulated tissues *in vivo*, including estrogen-stimulated breast cancers (10-13).

The physiological role of AFP, and especially tAFP, may be to act as a rudimentary servo mechanism that desensitizes endocrine tissues to the inappropriately high levels of steroid hormones that occur during gestation. This mechanism is fetoprotective, as the fetus develops in the presence of a large concentration of maternal and placental steroid hormones, and has receptors for these hormones, but it does not have the sophisticated control mechanisms of late fetal or adult life to regulate the production of and response to these hormones. A "side effect" of the proposed "servo mechanism" would occur when tAFP crossed the placenta into the maternal circulation where it would extinguish microscopic premalignant and/or cancerous foci in the breast that later on in life would be promoted to clinically detectable breast cancers. Such a "side effect" would explain the epidemiological data, which clearly show that the experience of full-term pregnancy decreases the lifetime risk of breast cancer (14).

Purpose: The purpose of our study is to produce large quantities of the active form of AFP and assess its effectiveness in the control of estrogen-stimulated growth of experimental human breast cancers.

The **specific aims** of our original grant proposal were:

1. Determine optimal conditions for producing the active form of AFP. Then, maximize the antitumor activity of AFP by manipulating its dose and schedule without introducing host toxicity in mice bearing human breast cancer xenografts.
2. Determine markers on tumors which predict tumor sensitivity to AFP.
3. Determine intermediate markers in the host which indicate that AFP is active *in vivo*.
4. Assess through histomorphometric studies the type of damage (lethal or non-lethal) done to the tumor by AFP.

These aims are specifically designed so that, upon their completion, the tools will be available for clinical trial of AFP for breast cancer.

Background (Taken from year 2 progress report): There are experiments of nature and laboratory experiments which point to AFP as a regulator of estrogen-stimulated growth of normal and malignant tissues. This has implications for AFP in the prevention and treatment of breast cancer, because almost all breast cancers start out as estrogen-receptor-positive and are stimulated in their growth by estrogen. By the time breast cancer is diagnosed, half of these breast cancers have further dedifferentiated to an estrogen-receptor-negative phenotype.

The evidence which supports the idea that AFP inhibits the response of tissues to estrogen is as follows. It is a well known fact that hepatomas secrete AFP (15). In fact, serum AFP levels are used as a marker of tumor burden in this disease. What is less well known is that amenorrhea is one of the first symptoms of hepatoma in premenopausal women and this symptom resolves following surgical removal of the tumor (16). Also, hyperestrogenemia and normal to elevated gonadotropins are present in hepatoma patients (17). Taken together, these data suggest that neither the uterus nor the hypothalamic-pituitary axis are responding to estrogen in hepatoma patients. It is our belief that elevated AFP levels could bring about this result. Data which support the contention that AFP can interfere with estrogen-dependent responses are as follows. Our own studies have shown that an isoform of AFP, upon exposure to estradiol, takes on a conformation that inhibits the estrogen-stimulated growth of normal mouse uterus (7). Soto et al. (18) have shown that AFP-containing serum from a hepatoma-bearing rat inhibits the estrogen-stimulated induction of progesterin receptor. These same investigators have shown that an AFP-secreting tumor induces the regression of an estrogen-dependent tumor (19). Epidemiological data suggest that AFP is the factor in pregnancy which confers on parous women their significant reduction in risk of breast cancer. As shown in Table 1a, AFP is elevated in maternal serum during pregnancy. Furthermore, there are factors in pregnancy such as maternal race, weight, hypertension, consumption of alcohol, number of fetuses *in utero*, and neural tube defect in the fetus where maternal serum AFP (MSAFP) is substantially altered from normal pregnancy levels. In studying the literature, we have found the consistent and striking correlation that in those pregnancy conditions associated with an elevated level of MSAFP, there was a significant reduction in the lifetime risk to the mother of acquiring breast cancer. Conversely, in pregnancy conditions characterized by low MSAFP (alcohol), subsequent breast cancer risk was elevated (Table 1a). We carried out epidemiologic studies analyzing retrospective data that extend and confirm the correlation between MSAFP levels and breast cancer risk (Table 1b). Recently Ekblom et al. (33) have published an epidemiological study which suggests that, at least in the case of hypertension during pregnancy, the reduction of breast cancer risk is also passed on to the fetus. He is in agreement with our speculation that it is AFP in the fetal and maternal circulation that protects the offspring as well as the mother against later development of breast cancer.

Table 1
Association of High Maternal Serum AFP with Decreased Breast Cancer Risk

Maternal Conditions		Maternal Serum AFP Concentration		Maternal Lifetime Breast Cancer Risk
1	2			
1a				
Pregnant	vs. Non-pregnant	1 > 2	(20)*	1 < 2 (21)
Pregnant, black	vs. Pregnant, white	1 > 2	(22)	1 < 2 (23)
Pregnant, lean	vs. Pregnant, obese	1 > 2	(22)	1 < 2 (24)
Pregnant, consuming no alcohol	vs. Pregnant, consuming alcohol	1 > 2	(25)	1 < 2 (26)
1b				
Pregnant, hypertensive	vs. Pregnant, normotensive	1 > 2	(27)	1 < 2 (28)
Pregnant, with multiple fetuses	vs. Pregnant, with a single fetus	1 > 2	(29)	1 < 2 (30)
Pregnant, fetus with neural tube defect	vs. Pregnant, fetus no neural tube defect	1 > 2	(31)	1 < 2 (32)

*The numbers in the brackets are the reference sources for the data.

Recently, Richardson et al. (34) have reported measuring this association directly. She found that the concentration of AFP in cryogenically stored maternal sera was inversely correlated to the risk of breast cancer in these mothers 20 to 30 years after their pregnancies. As mentioned earlier in this report, we speculate that the AFP which crosses the placenta and enters the maternal circulation extinguishes microscopic premalignant foci that later on in life would be promoted into clinically detectable breast cancers. Our own work has shown that administration of natural mouse AFP (10), natural human AFP (11), or recombinant human AFP (13) can inhibit estrogen-stimulated growth of human MCF-7 breast cancer xenografts.

In year one of this grant we focused our study on recombinant human AFP obtained from our collaborators at McGill University, who licensed the rights to their patent for producing AFP to a start-up biotech company, Atlantic Biopharmaceuticals. We tested several batches of their AFP and found variable activity (described in detail in year 2 progress report). We believe the basis for this variability was the harsh conditions used to isolate the protein from the *E. coli* expression system. The protein was not secreted in this system. Rather, it was contained in inclusion bodies inside the *E. coli*. The chemical conditions required to lyse the *E. coli* and break the inclusion bodies denatured the protein. The resolubilized protein was diluted and allowed to refold but in some cases probably did not return to the activable conformation. In retrospect, an expression system which secreted the protein would be preferable, and we took advantage of this experience and developed two AFP secretory systems in year 2 of the grant. One system was recombinant AFP domain III produced in a baculovirus expression system. The second system was natural

AFP produced by a human hepatoma (Hep G-2) in serum-free cell culture. Based on the information described in the background section on page 6, we surmised that the role of the AFP-activating ligand, such as estradiol, was to create a chemical environment which fixed more of the AFP molecules in their active form. This suggested to us that some of the AFP molecules were already in their active form, and if we were to raise the dose of AFP, we could reach an activity level similar to that obtained with ligand-transformed AFP. We tested this hypothesis in the latter part of year 2 of the grant and found that this indeed was the case. Both natural AFP isolated from human hepatoma cells and recombinant AFP-domain III inhibited estrogen-dependent growth if given at doses of 10 μ g or higher. Thus, increasing the dose of AFP exempts the protein from the ligand-induced activation requirement, simplifies its use, does not introduce toxicity and, in our opinion, makes it more translatable to the clinic.

We had spent a significant amount of time during the first two years of the grant trying to develop the optimal form of AFP to use against the spectrum of human tumor xenografts which we had outlined in our proposal. Further progress can still be made in developing the optimal form of AFP, and some of this work will be described later in the report (see page 28). However, at this stage of the research we needed to choose a form of AFP which could be used to complete the remainder of the work and test the principles laid out in this project as well as establish the potential usefulness of AFP as a treatment for breast cancer. Based on criteria such as ease and economy of production, yield, stability, activity and storability, we concluded that natural AFP secreted by Hep G-2 cells was the preparation that we should proceed with. Most of the work in year 3 of the grant was carried out with this particular preparation of AFP. However, as described later in this report, we also made progress with forms of AFP truncated down from domain III and peptides that were selected and synthesized based on our truncated forms of AFP.

BODY

a. Methods and Results Obtained

1. Activity of human AFP (Hep G-2-derived) against human cancer xenografts.

Several human breast and non-breast cancer cell lines were grown as a monolayer in culture. To prepare these cell lines for growth as xenografts, confluent monolayers were trypsinized, pelleted by centrifugation, and solidified by exposure to 15 μ l of fibrinogen (50 mg/ml) and 10 μ l of thrombin (10 units/ml) for 30 minutes at 37°C. Fibrin clots were then cut into pieces approximately 1.5 mm in diameter, and each piece was implanted under the kidney capsule of severe combined immunodeficient (SCID) mice (35, 36). For estrogen-dependent tumors, mice were supplemented with a Silastic tubing implant containing estradiol (Si/E₂), which was placed subcutaneously (37). AFP was injected i.p. once a day for the times indicated in the figures. There were 5 to 10 replicate mice per treatment group.

As shown in Figures 1 and 2, the MCF-7 breast cancer was dependent on estrogen for its growth. Significant growth inhibition was obtained with 10 μ g of AFP and complete growth inhibition was achieved with 100 μ g of AFP. A dose of 100 μ g of AFP also stopped the growth of MCF-7 tumors which had been allowed to seed and grow for 10 days prior to treatment

(Fig. 3). Upon cessation of AFP treatment, tumor growth resumed (Fig. 3). This pattern of growth regulation was similar to that found with tamoxifen in this model (Fig. 3b). Albumin, given at a dose of 250 µg per mouse per day, did not inhibit the growth of MCF-7 breast cancer xenografts (Fig. 3b).

The T-47 D human breast cancer was dependent on estrogen for growth in our model (Fig. 4). AFP given daily completely stopped the growth of this tumor (Fig. 4). AFP also stopped the growth of T-47 D tumors which had been allowed to seed and grow for 10 days prior to treatment (Fig. 4). Again, upon cessation of AFP treatment, tumor growth resumed.

The MDA-MB-231 and BT-20 human breast cancers grew in an estrogen-independent manner in our xenograft model. Neither of these tumors were inhibited by either AFP or Tamoxifen (Figs. 5 and 6) when these agents were given at the dose and schedule which inhibited the estrogen-dependent human breast cancers described above.

We are currently working on the ZR-75-1 (estrogen-receptor-positive) and the HS 578 T (estrogen-receptor-negative) human breast cancers. We are having difficulty growing these tumors as xenografts. We have also had difficulty growing as xenografts the two freshly resected patient breast cancers which we have obtained as part of this project. Strategies to overcome this difficulty include using matrigel in the tumor inoculum, changing the level of hormone supplementation, and changing the host mouse to a NOD SCID mouse, which has been reported to have a lower level of natural killer cells than the ICR SCID mouse (38).

We have also investigated the sensitivity of non-breast cancers to AFP. The LNCaP human prostate cancer was dependent on androgen supplementation (testosterone propionate, 0.4 mg/mouse s.c. every other day) for its growth (Fig. 7). This tumor was completely stopped in its growth by daily administration of 100 µg of AFP. In contrast, the DU 145 human prostate cancer did not require androgen supplementation for growth and was not inhibited by AFP (Fig. 8).

The OVCAR-3 human ovarian cancer did not require hormone supplementation for growth and grew in a cyst-like pattern. Growth was slightly retarded in the presence of AFP, but the differences were not significant (Fig. 9). Similarly, the MFE-296 human endometrial cancer did not require hormone supplementation for growth and was not inhibited by daily treatment with AFP (Fig. 10).

The histopathology of tumors growth-inhibited by AFP continued to be a profile of cytostasis with an accumulation of cells in G₀G₁ and no evidence of necrosis. This was described in more detail in the year 2 progress report. Also described in our year 2 progress report was our ancillary study (supported by separate funding from Dr. Bruce Line, Chief of Nuclear Medicine at Albany Medical College) using technetium-labeled AFP to image human breast cancer xenografts. It should be noted here that in that study technetium-labeled AFP continues to provide higher tumor-to-background ratios and clearer images than technetium-labeled sestamibi, which is in clinical trials for this purpose. Moreover, tumor-to-background ratios with technetium-labeled AFP were similar in both AFP-growth-inhibitable and AFP-non-growth-inhibitable tumors, suggesting that AFP was getting into non-growth-inhibited tumors. Since the AFP-growth-inhibitable tumors were those which were dependent on hormone for growth, it further suggests that growth inhibition occurs as a result of AFP interfering with some step in the biochemical pathway responsible for hormone-dependent growth and is not based on AFP uptake by the tumor.

Fig. 1

MCF-7

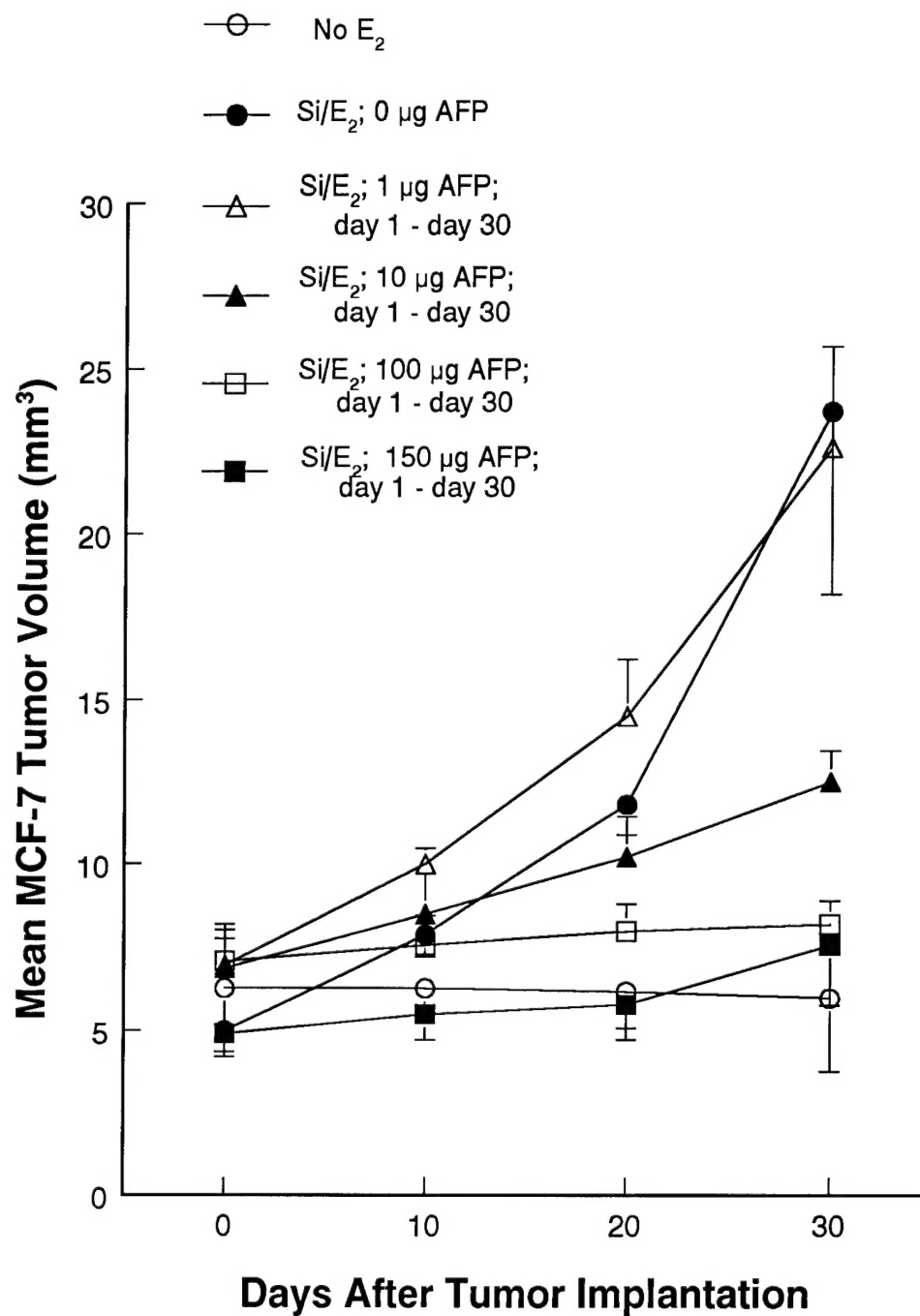


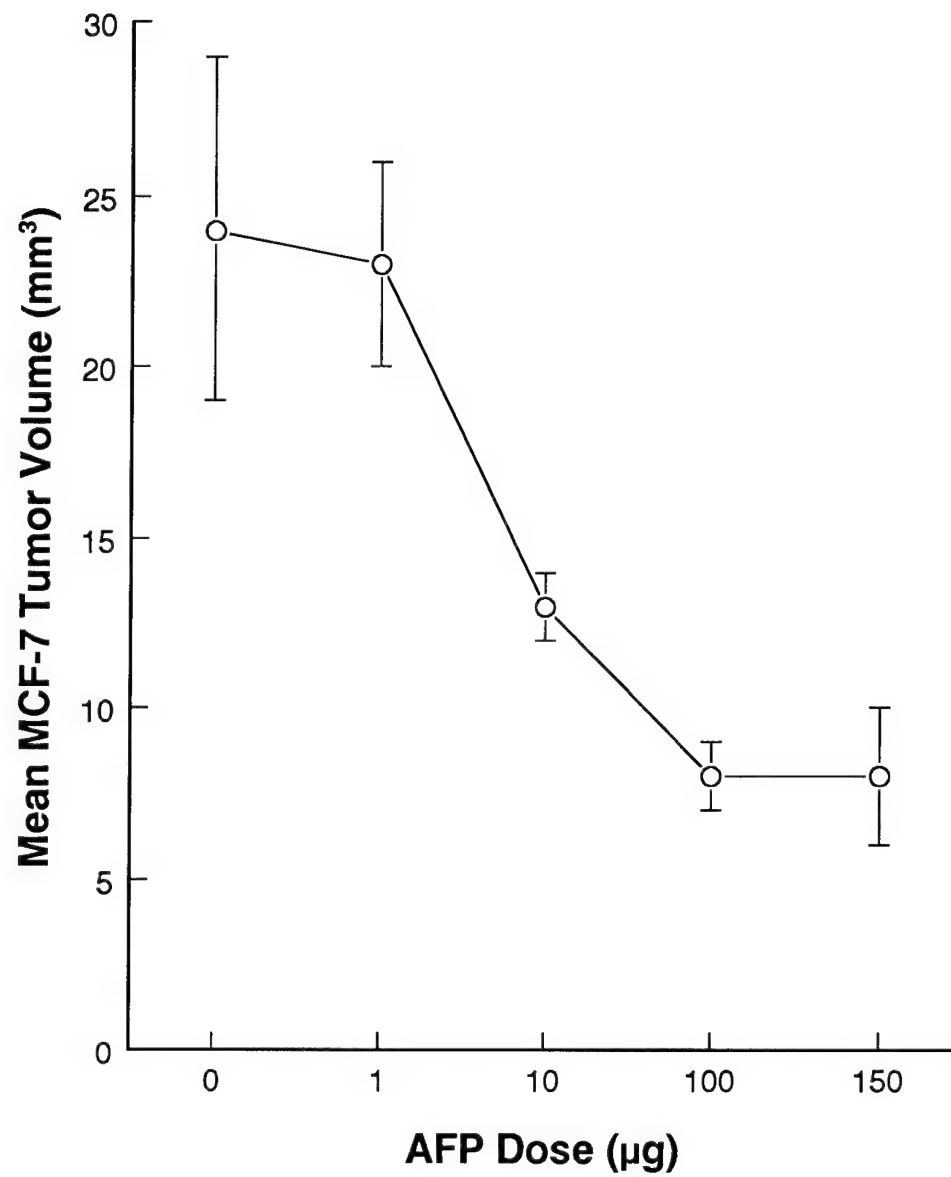
Fig. 2**MCF-7**

Fig. 3

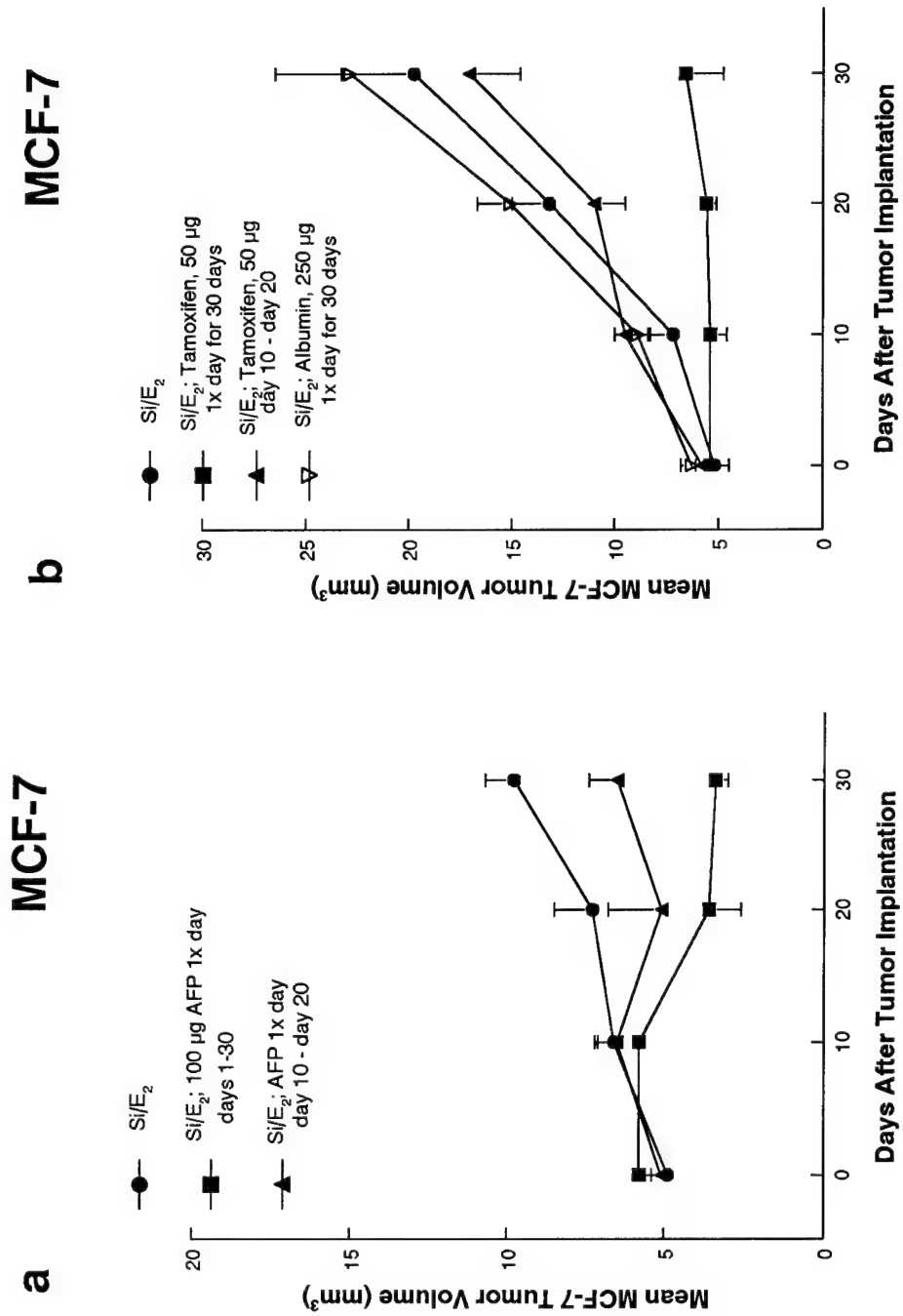


Fig. 4

T47

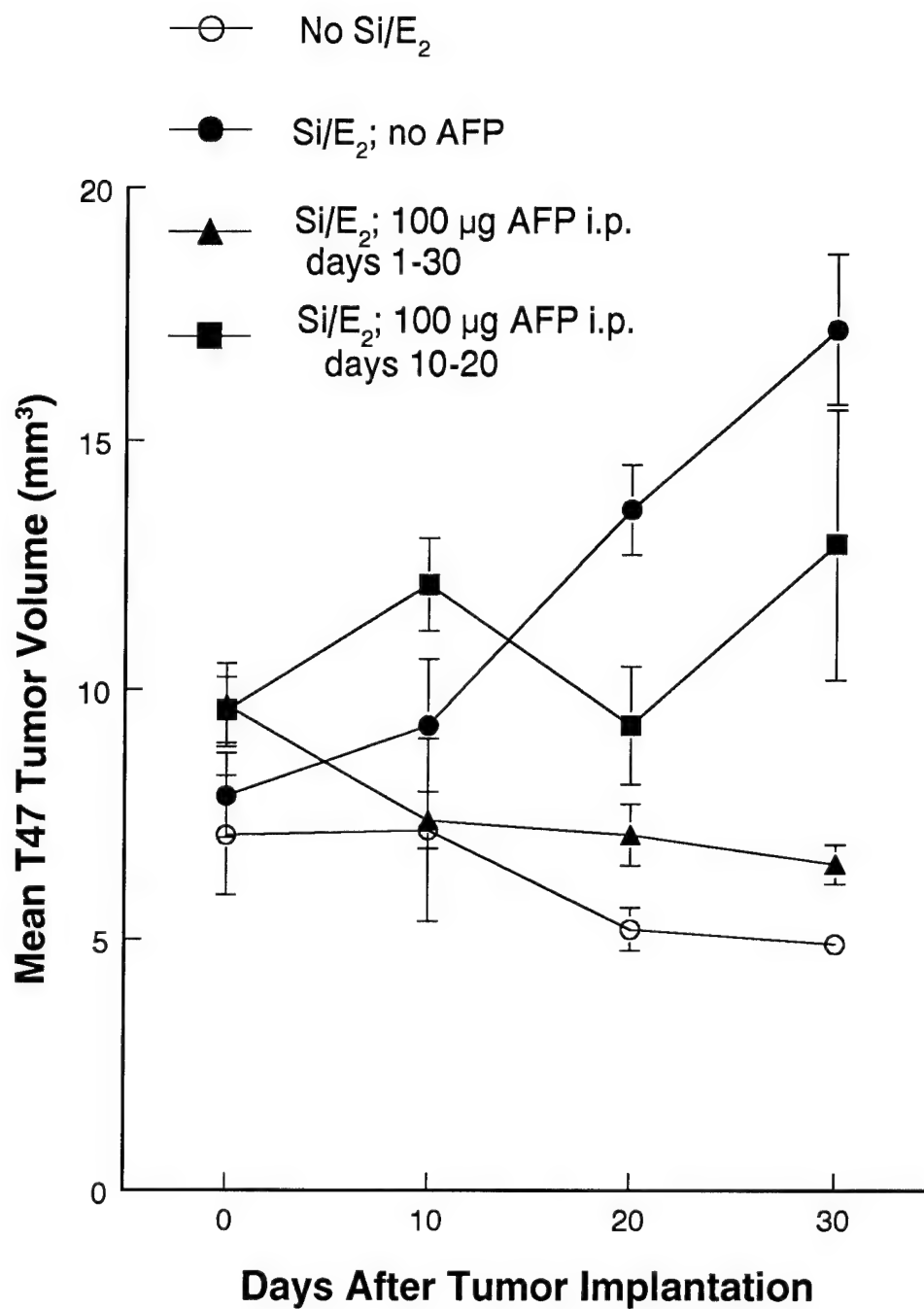


Fig. 5

MDA-MB-231

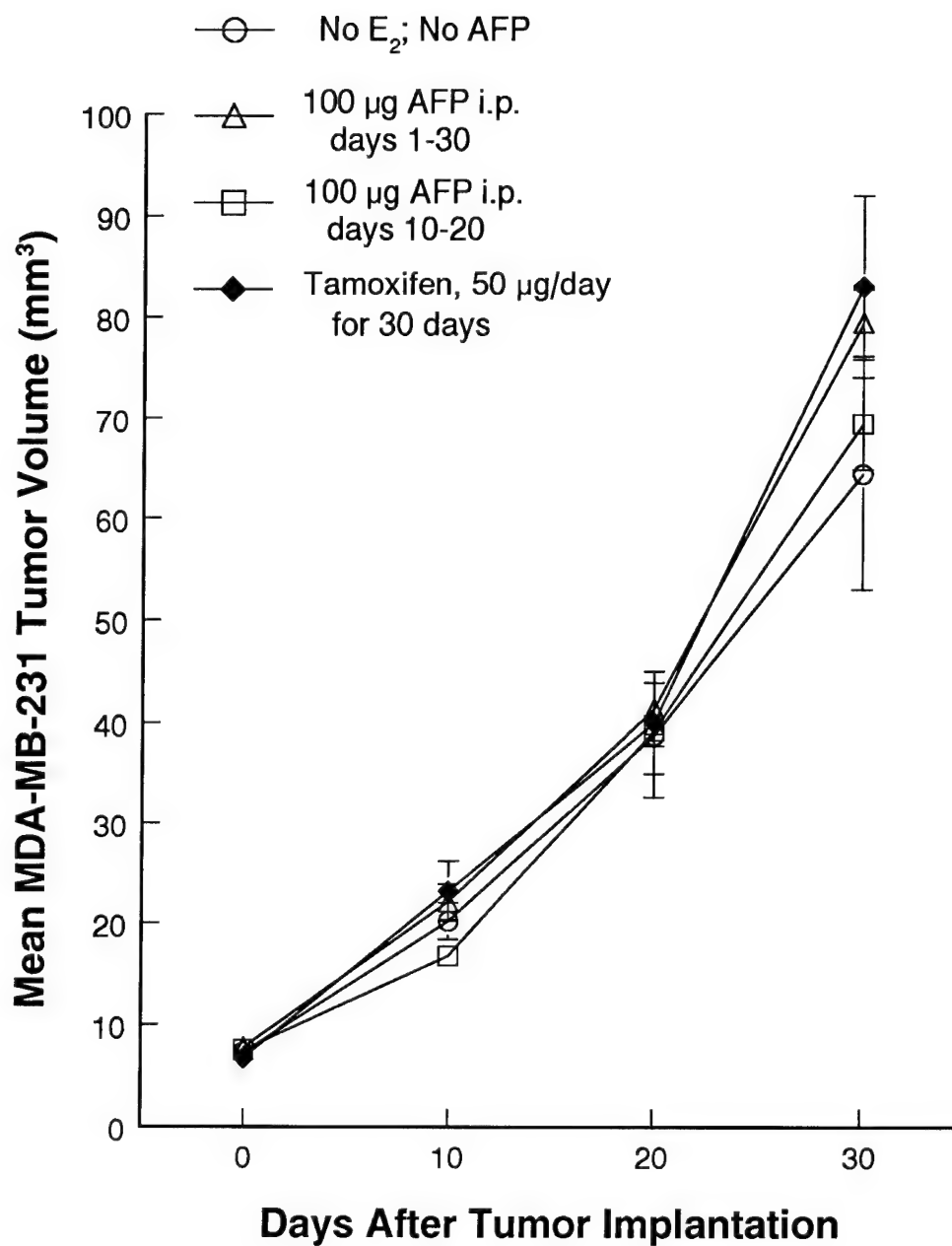


Fig. 6

BT-20

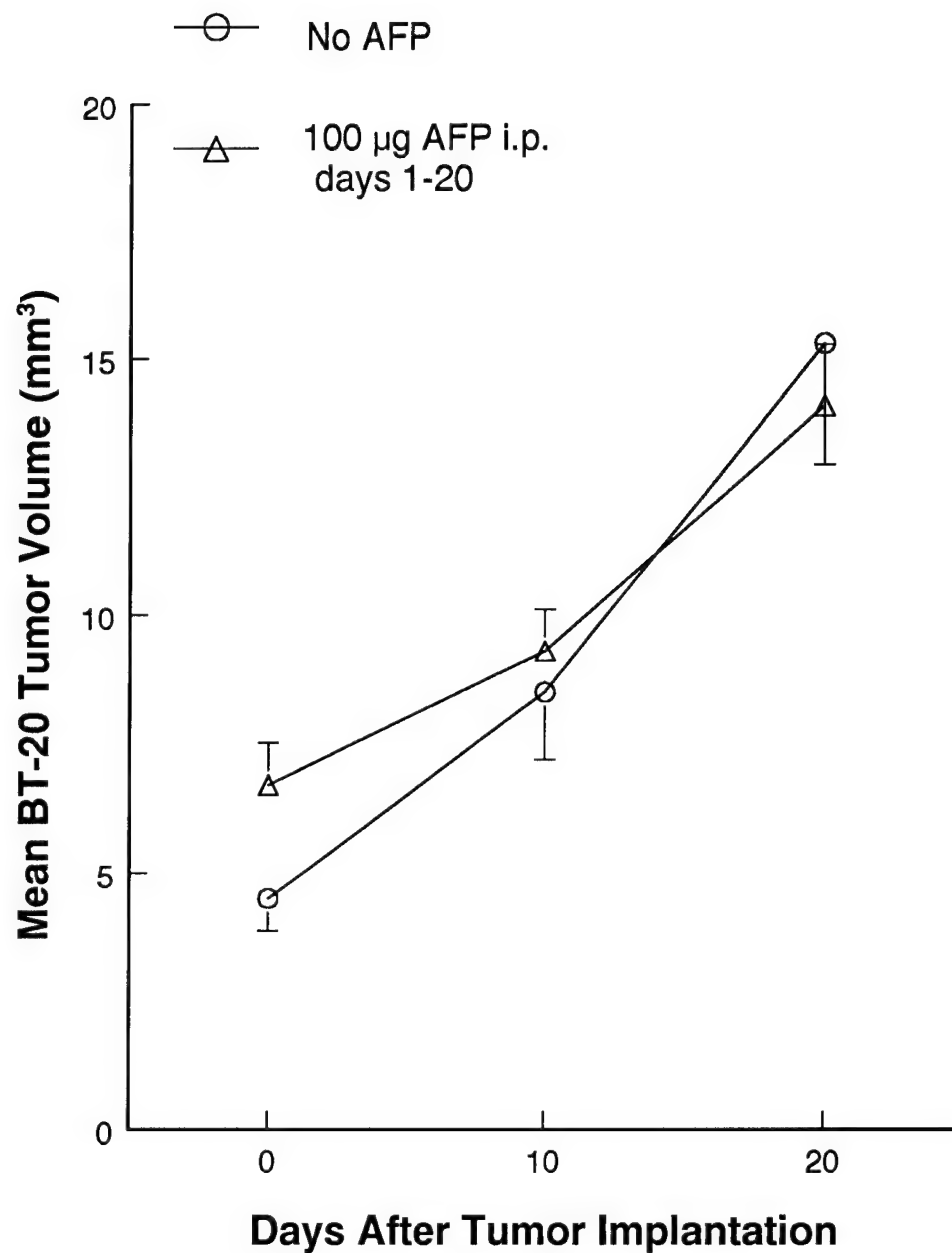


Fig. 7

LNCaP

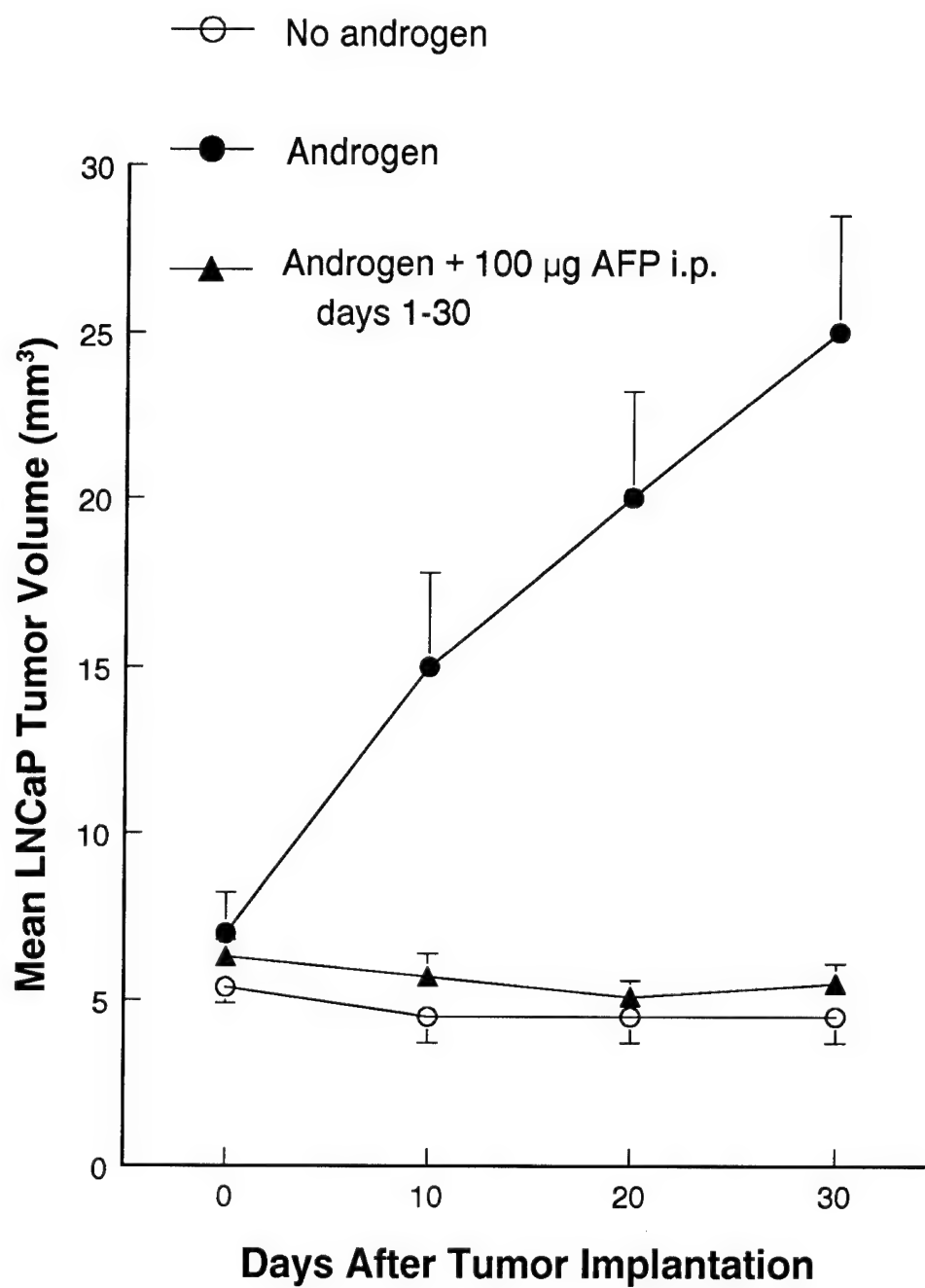


Fig. 8

DU 145

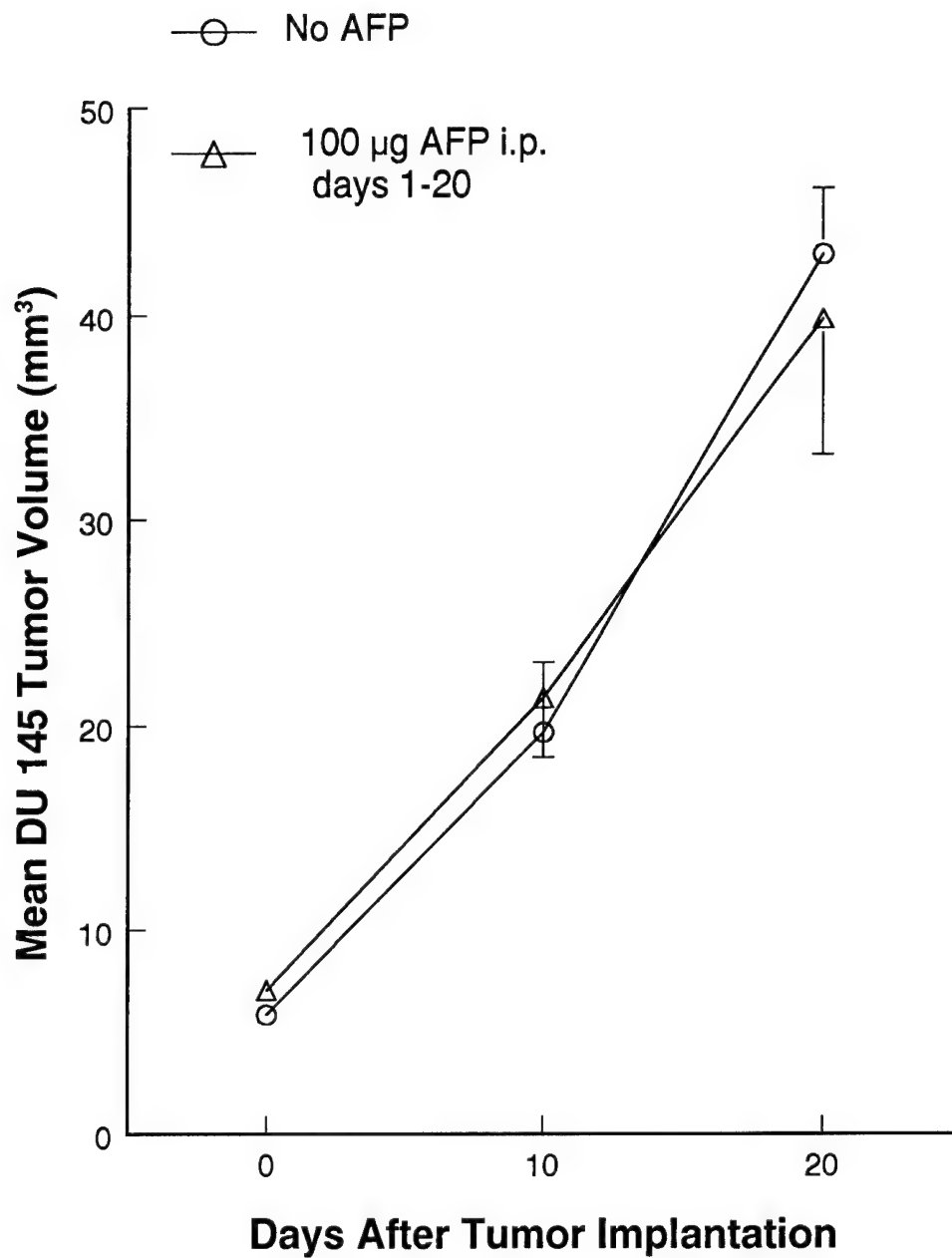


Fig. 9

OVCAR-3

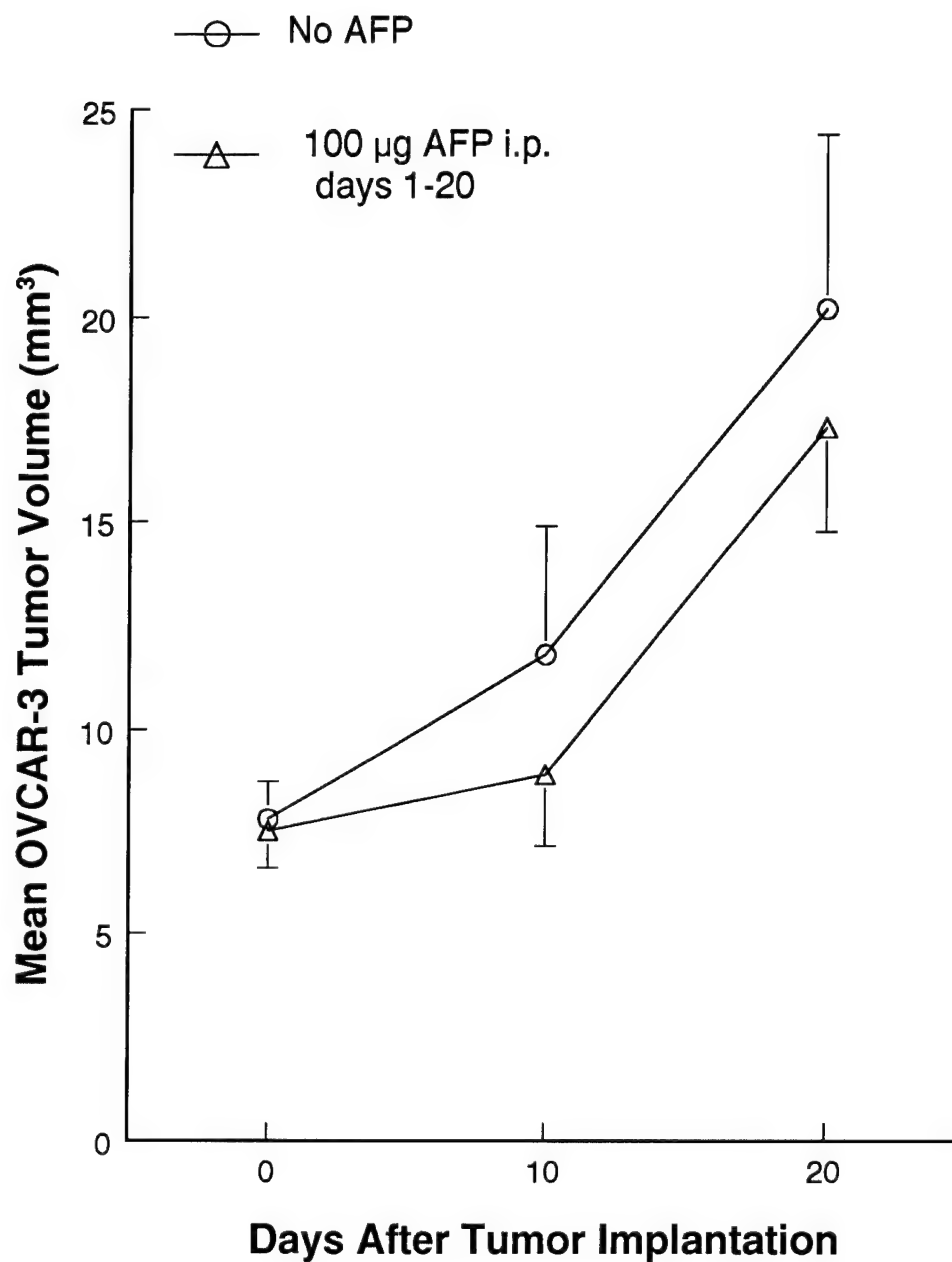
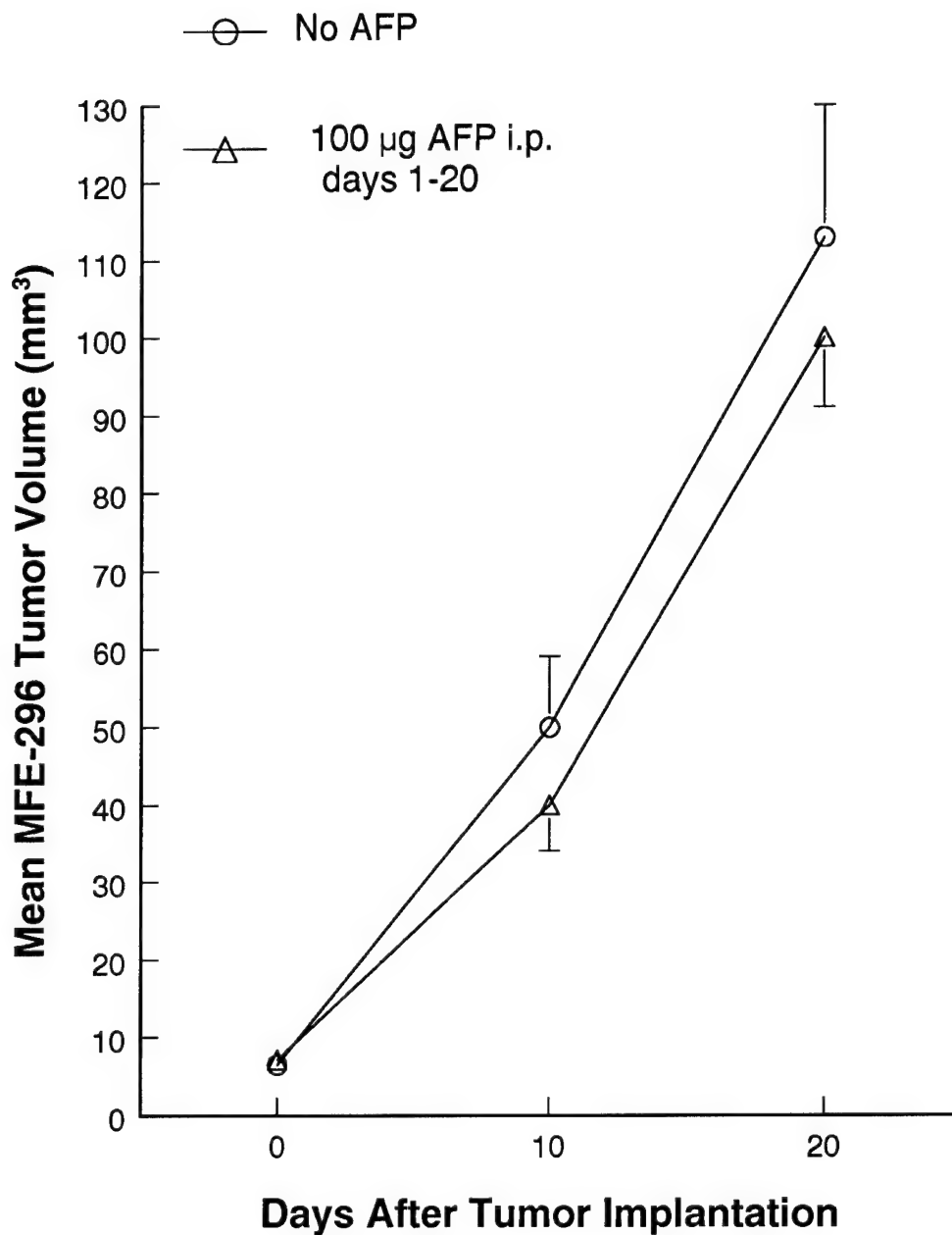


Fig. 10

MFE-296



2. Markers on Tumors Which Predict Tumor Sensitivity to AFP

a. Sex steroid hormone receptors

Cells were grown in bulk culture, harvested by trypsinization, pelleted, resuspended in buffer and stored at -70°C until used. All three receptor determinations (estrogen receptor [ER], progesterone receptor [PR] and androgen receptor [AR]) were done at one time, and required 600 mg of each cell line to yield sufficient cytosol volume and protein concentration. Aliquots of cells were thawed, homogenized and centrifuged at 60,000 rpm for one hour at 4°C . Supernatant (cytosol) protein concentration was determined using BioRad (Bradford) protein assay, and adjusted to between 2.0-2.5 mg/ml in a final volume of 6.0 ml.

Six-point Scatchard plot analyses were performed for each of the receptors at the following concentrations of radioligand: 0.1, 0.5, 1.0, 2.0, 4.0 and 8.0 nM with 12.5 μM radioinert steroid as inhibitor. ER was determined using ^3H estradiol (E_2) and unlabeled E_2 ; PR with ^3H progesterone and 6 α -methyl-17 α -hydroxy progesterone acetate (MPA); and AR with ^3H dihydrotestosterone (DHT) and DHT. Samples were assayed in duplicate at 4°C .

Tubes used to assess total counts contained only labeled steroid in buffer. Tubes used to assess total binding contained 100 μl cytosol and labeled steroid. Tubes used to assess non-specific binding contained the same as specific-bound tubes, but with the addition of a molar excess of unlabeled steroid as inhibitor. Specific binding was calculated as the difference between total binding and non-specific binding.

Controls were performed for every assay using rabbit kidney cytosol as negative control for all three receptors, rabbit uterus cytosol as positive control for ER and PR, and castrated rat prostate cytosol as positive control for AR.

After overnight incubation all tubes except total counts were stripped of unbound steroid with charcoal. Following centrifugation at 1500 rpm at 4°C , supernatants containing bound steroid were decanted into scintillation vials for counting.

The sex steroid receptor values for the various tumor cell lines are shown in Table 2.

Table 2

Tumor Line	ER	PR	AR
	(fmol/mg cytosol protein)		
MCF-7	17	96	89
T-47 D	16	151	30
ZR-75-1	0	233	34
MDA-BM-231	0	22	0
BT-20	0	14	0
Hs 578 T	0	0	0
LNCaP	0	0	560
DU 145	0	0	0
OVCAR-3	65	0	19
MFE-296	0	0	37

The tumors which were dependent on hormone for growth (MCF-7, T-47 D, LNCaP) were clearly positive for the hormone receptors corresponding to the hormone stimulating their growth. Similarly, the tumors which grew independent of hormone (MDA-MB-231, BT-20, DU 145, OVCAR-3, MFE-296) with the exception of OVCAR-3 were clearly negative for sex steroid hormone receptors. Growth conditions are still being developed for ZR-75-1 and Hs 578 T. Surprisingly, supplementation of SCID mice with estrogen, progesterone, androgen or estrogen plus progesterone did not result in consistent growth of ZR-75-1. Also, Hs 578 T, which is a very dedifferentiated tumor, has not grown well in either SCID or nude mice. Both of these tumors have been reported by the ATCC to grow as xenografts in nude mice. Since SCID mice are more immunosuppressed than nude mice, and since tumors generally grow as xenografts better in SCID mice than in nude mice, it is not clear to us why we have had difficulty growing only these two particular tumors. We are currently trying to resolve this dilemma.

b. AFP Receptor Assays

Dual approaches were developed to assay AFP receptor. One approach was based on a report by Villacampa et al. (39) which utilized radiolabeled AFP, and the other approach was based on work by Moro et al. (40) which required only radioinert AFP and monoclonal antibody to AFP receptor.

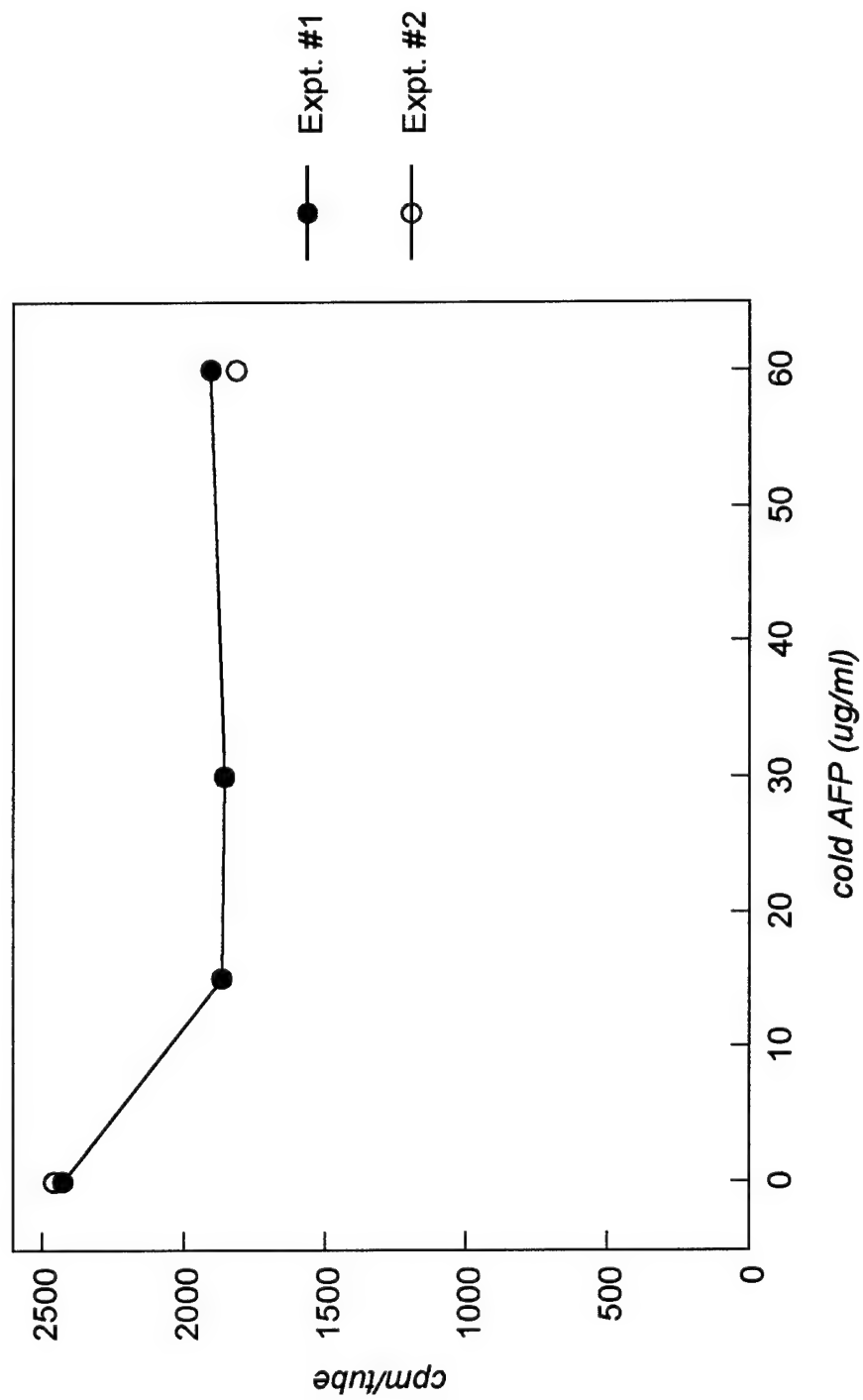
As described by Villacampa (39), we labeled AFP with ^{125}I using the Iodogen technique (41). The resultant specific activity of ^{125}I AFP was $1.49 \mu\text{Ci}/\mu\text{g}$. MCF-7 cells were added to siliconized, albuminized microcentrifuge tubes at 10^6 cells in 0.1 ml medium with 1% serum and kept at 4°C throughout the procedure. Radiolabeled AFP was added in the presence and absence of excess cold AFP, and all tubes were brought to a final volume of 0.2 ml. Tubes were incubated 4 hours at 4°C , after which the cells were washed twice with medium and once with PBS. The bottom portion of each tube, containing the cell pellet, was cut and placed in a vial, which was then counted in a gamma counter.

Villacampa (39) used 500-1,000-fold excess cold AFP to saturate specific AFP binding sites. We felt this was an excessive amount of AFP (milligram amounts) to use in a routine assay, and it would rapidly deplete our stores of AFP. Therefore, our first experiments were designed to determine the minimum amount of cold AFP needed to block specific sites during an overnight incubation prior to the addition of labeled AFP. We assessed various concentrations of cold AFP ranging from two- to seventy-fold excess over hot material. As shown in Figure 11, a three-fold excess of cold AFP in an overnight preincubation was sufficient to saturate specific binding sites. In spite of these data, our Scatchard plots did not reproduce the affinity constant nor the total number of receptor sites described by Villacampa (39).

Concurrently we were developing an alternative, non-radioactive method using monoclonal antibody to AFP receptor to block specific sites. For this method MCF-7 cells at 2.5×10^6 cells in 0.1 ml were added to microcentrifuge tubes. AFP was added in the presence or absence of monoclonal antibody, and all tubes were brought to a final volume of 0.2 ml and kept at 4°C during the binding phase of this procedure. Cells were incubated with either antibody to AFP receptor or isotype control serum for 30 minutes, after which AFP was added. After an additional

3-hour incubation, cells were washed 3 times and then 20 nM sodium azide was added for 5 minutes to metabolically arrest cells to prevent internalization of AFP receptor complex in subsequent steps. KCl (0.4 M) was added for one hour at 37°C to dissociate AFP from its receptor (42). Cells were centrifuged and supernatant was evaluated for AFP content. As shown in Fig. 12, KCl does liberate AFP from its receptor. Also, as shown in Fig. 13, monoclonal antibody to AFP receptor decreases the amount of KCl-recoverable, cell-associated AFP. We are currently engaged in experiments varying the concentrations of AFP in the presence and absence of saturating inhibitor (i.e., monoclonal antibody to AFP receptor) and will be generating Scatchard plots from this data. We are encouraged by this alternative method in comparison to the Villacampa method, because it is completed in less time, utilizes substantially less AFP, and does not require radioactive materials.

**Fig. 11. SATURATION OF MCF-7 AFP RECEPTOR
SITES BY EXCESS COLD AFP**



* I-125 AFP was 5.6 ug/ml and 0.028 uCi/ug in all tubes

Fig. 12.
AFP RECOVERED FROM MCF-7 CELLS
FOLLOWING KCI EXTRACTION

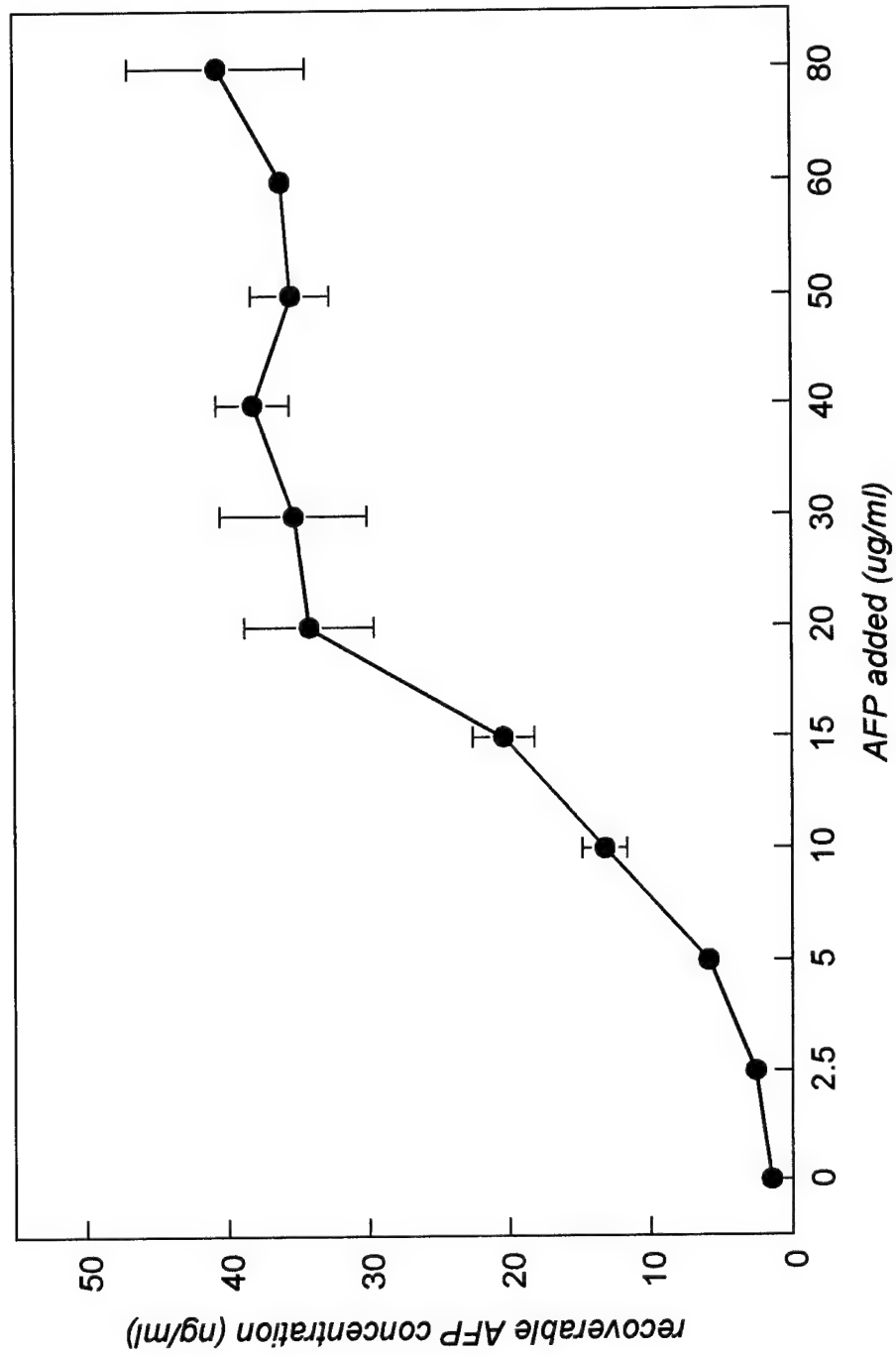
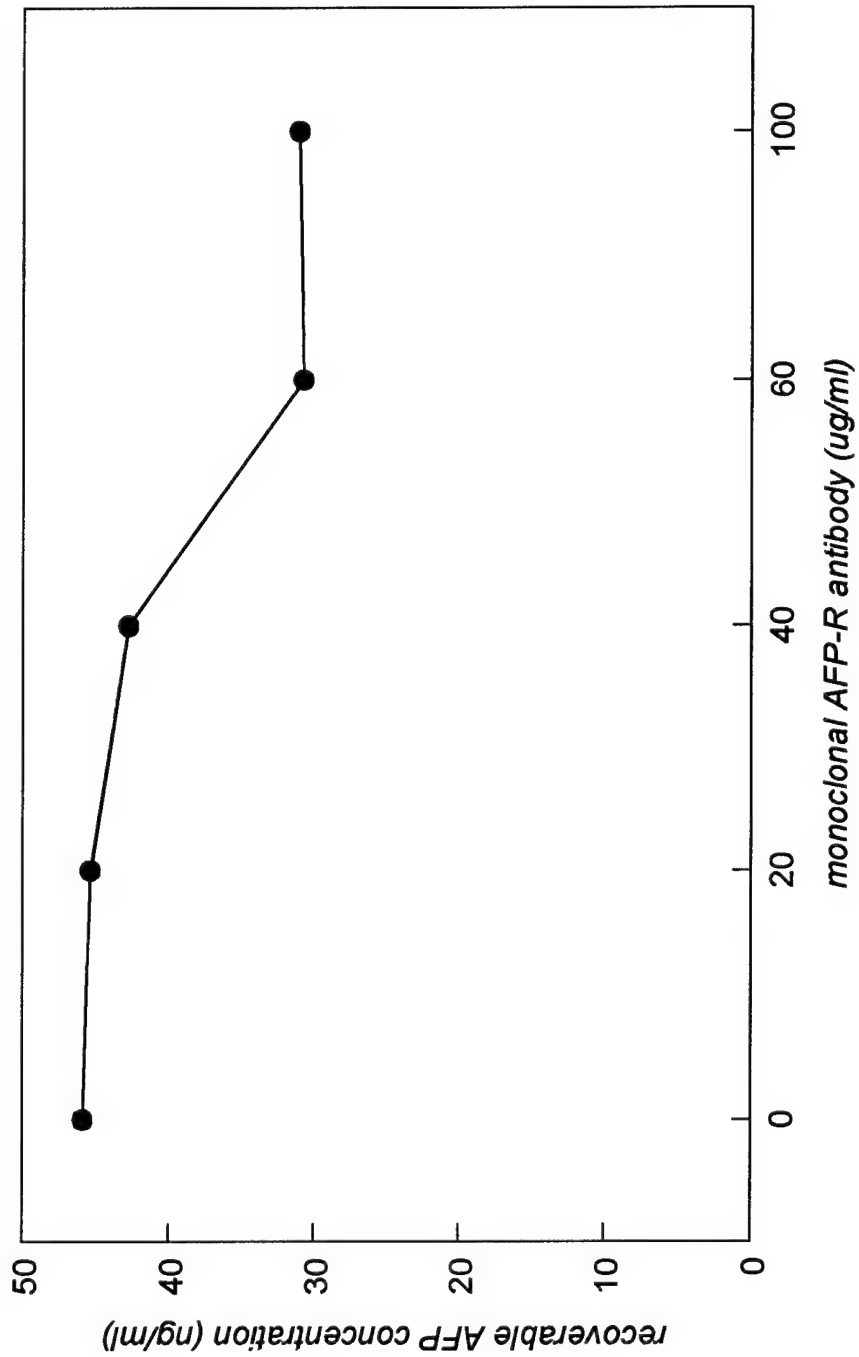


Fig. 13. SATURATION OF MCF-7 AFP RECEPTOR BY MONOCLONAL ANTIBODY TO AFP RECEPTOR



* AFP was 30 ug/ml in all tubes

3. Intermediate Markers in the Host Which Indicate That AFP is Active *in Vivo*

a. Gonadotropin Assay

Radioimmunoassay Reagent Preparation. LH and FSH levels were estimated by double antibody RIA using reagents from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). LH and FSH preparations (NIDDK-rLH-I-9 and NIDDK-rFSH-I-8) were iodinated by the chloramine-T method (43). Briefly, LH or FSH iodination preparations were thawed, and one millicurie of ^{125}I was added to the tube and mixed. Chloramine-T was added (25 μl of a 1 mg/ml solution), and this solution was mixed and allowed to react for 2 minutes at room temperature. Sodium metabisulfite (50 μl of 2.4 mg/ml in 0.05 M PBS) was then added to quench the reaction. In order to separate labeled protein from free ^{125}I , this solution was gently layered on top of a gel surface (G-75 Sephadex column) and allowed to run into the column. The column was then continuously washed with 1% BSA in PBS until 15-20 1-ml fractions were collected. Aliquots of 5 μl were counted. The first radioactive peak was selected as labeled hormone, and the second peak was discarded as free ^{125}I .

The fractions making up the peak of iodinated hormone were frozen, and the one with the greatest count was utilized in the radioimmunoassay. The "cold standards" (NIDDK-rLH-RP-3 and NIDDK-rFSH-RP-2) were reconstituted with 1 ml of distilled water to yield a solution of 5 $\mu\text{g}/\text{ml}$ in 1% BSA. The reference protein was aliquoted at 25- μl volumes and frozen for later use in assays. The first antibodies for LH and FSH (NIDDK-anti-rLH-S11 and NIDDK-anti-rFSH-S-11) were reconstituted in 1 ml of distilled water, aliquoted and stored frozen. Final dilutions of all NIDDK reagents were conducted just prior to use in assays.

Radioimmunoassay Procedure. All NIDDK reactants were added to RIA tubes in one sitting, at refrigerator temperature, in the sequence: a) buffer, b) "cold standard" or unknown, c) radioiodinated hormone (NIDDK-rLH-I-9 or NIDDK-rFSH-I-8), d) antiserum (1:750,000 dilution for anti-LH and 1:125,000 dilution for anti-FSH). The reactants were then incubated at room temperature for 24 hours. The "second" antibody (goat anti-rabbit IgG, Sigma # R-5506) was added after this 24-hour incubation and allowed to incubate for another 24 hours. The following morning 0.5 ml of 25% polyethylene glycol was added to each tube, then tubes were vortexed and allowed to incubate for 15 minutes. All tubes were centrifuged at 1000 x g. The supernatants were aspirated and the pellets counted in a Beckman Crystal Plus gamma counter. Specific binding was obtained by subtracting non-specific binding (tubes pipetted with tracer, buffer, and second antibody, omitting first antibody) from total binding. Standards and unknowns were calculated as a percent of specific binding based on their competitive reduction of that binding. Unknowns were assayed in duplicate and calculated from a weighted logit linear regression treatment of the standard curve. Standard curves were prepared in triplicate and covered a range of 7.8-500 ng/tube. Unknowns were assayed in duplicate and diluted so as to be read near the middle of the standard curve. This allowed the use of the more linear portion of the curve (25-250 ng/tube). The ED_{50} for LH was approximately 123 ng/tube, and the ED_{50} for FSH was approximately 115 ng/tube. Specific binding was calculated to be 30% and 45% for FSH and LH, respectively. The coefficient of variance between replicates did not exceed 15%.

Trial-and-Error Component. Difficulties were encountered in establishing workable standard curves. Iodinations were conducted at the Albany Medical Center core facility. Initial iodinations of gonadotropins resulted in low specific binding of FSH to its antibody (14%) and no binding of LH to its antibody. It was felt that the low and absent binding was due to harsh iodination conditions that possibly destroyed or significantly altered the tertiary structure of the gonadotropin molecules. Both gonadotropins had extremely high specific activities. New strategies were developed to reduce the specific activity of the gonadotropins and to increase the specific binding of gonadotropin to its respective antibody. It was decided that iodinations would be carried out utilizing iodogen compound, which would result in milder conditions that would conserve the integrity of the molecules. This procedure was conducted with both gonadotropins on two additional occasions. Lower specific activities were achieved; however, specific binding ranged between 2%-10%.

At this time we began to suspect that our problem was with the first antibody concentrations. In order to test this hypothesis, binding curves utilizing ten different levels of first antibody were set up, and specific binding was examined utilizing the hormone iodinated with iodogen. These curves also yielded low specific binding. We then obtained an anti-ovine LH antibody that had successfully been utilized in a rat LH assay set up at the New York State Department of Health. Again, ten LH binding curves were assayed in an attempt to discover a titre that would yield between 30%-40% specific binding. This attempt was also unsuccessful.

At this time we decided to go back to our original plan for iodination and obtain all new reagents from the NIDDK. Iodinations of the NIDDK-provided antibody were conducted utilizing lower concentrations of chloramine-T (approximately 10 µg/1 µg of antigen). Binding curves were set up using four different dilutions in an attempt to establish the appropriate antibody titre. Finally, adequate specific binding was obtained using the NIDDK antibodies at a slightly lower final tube dilution and with antigen iodinated with the chloramine-T concentrations suggested by the NIDDK. Specific binding for LH was 45% and 30% for FSH.

We are beginning to apply this assay to assess gonadotropin levels in serum samples from tumor-xenograft-bearing mice receiving AFP. Female SCID mice bearing MCF-7 breast cancer xenografts were treated daily for 30 days with 100 µg of AFP. As previously shown in Figure 1, this treatment completely prevented tumor growth. Blood samples were taken from these mice at the end of the 30-day treatment interval. As shown in Table 3,

Table 3

Treatment	FSH (ng/ml)	LH (ng/ml)	E ₂ (pg/ml)
No E ₂ ; No AFP	127 ± 34	214 ± 88	56 ±
Si/E ₂ ; No AFP	89 ± 25	210 ± 76	100 ± 5
Si/E ₂ ; AFP 100 µg mouse/day	119 ± 22	203 ± 69	187 ± 8

supplementation of mice with Silastic estradiol (Si/E₂) implants increased the serum level of E₂ and decreased the level of FSH. This is consistent with an E₂ feedback mechanism on the hypothalamic pituitary axis down-regulating the output of FSH. AFP treatment prevented this

decrease in FSH and further increased serum E₂ levels. This is consistent with an inhibition by AFP of the estrogen-dependent feedback response on FSH secretion at the hypothalamic pituitary axis. There seemed to be no effect of either E₂ supplementation or AFP treatment on serum LH levels. We want to repeat these studies in male mice. Also, we want to look at changes in gonadotropin and androgen levels in male mice growing androgen-dependent LNCaP prostate cancer xenografts. At the time of this writing, it would appear that increases in serum gonadotropin and estrogen levels are intermediate markers which indicate that AFP is having its intended inhibitory effect on estrogen-dependent responses *in vivo*.

b. Breast-cancer-associated antigen assay

In our original proposal we postulated that a decrease in serum levels of tumor-associated antigen would be an intermediate marker indicating that AFP is having a direct inhibitory effect on the tumor. We reasoned that if AFP were stopping tumor growth, it would be slowing down the metabolic activity of the tumor, which should translate into less shedding of tumor-associated antigen. In our original proposal we indicated that we would measure serum levels of CA 15-3 in tumor-xenograft-bearing mice, since this tumor-associated antigen has been found to be elevated in serum of patients with breast cancer (44). However, upon further study of this issue, we have learned that CA 27.29 is more sensitive than CA 15-3 for detecting tumor presence (45). CA 27.29 is measured by a competitive inhibition radioimmuno-assay using the Truquant BR RIA kit purchased from Biomira Inc. (Canada). This is a solid phase RIA in which polystyrene tubes coated with CA 27.29 antigen are incubated with standards, normal serum controls or serum samples from tumor-bearing mice, then with ¹²⁵I-labeled BR 27.29 monoclonal antibody for 3 hours at room temperature. Tubes are washed twice with distilled water, and bound radioactivity in the tubes is measured in the gamma counter. This kit has been purchased, standard curves have been established, and we are ready to test our serum samples for this antigen in tumor-bearing mice with or without AFP treatment.

Continued Development of the Active Form of AFP

AFP is a glycoprotein consisting of 590 amino acids. Based on its structural homology to albumin, it has been divided into three domains (I, II, III) with each domain having three subdomains (A, B, C) (46). Through our collaboration with others and through our own work, we have determined that the active site of AFP is in a 34-amino-acid portion of Domain III B of the molecule. This was an arduous, stepwise task. Our collaborators from Japan provided us with Domain I of the molecule, and it was inactive in our estrogen-dependent growth assays. Our collaborators in Montreal provided us with Domain II-III of the molecule, and it was active in our estrogen-dependent growth assays. We produced Domain III in a baculovirus system and found that it was active in our estrogen-dependent growth assays. We then produced a portion of Domain III (Domain III AB, approximately 100 amino acids) in a baculovirus expression system and found that it was active in our estrogen-dependent growth assays. All of this work localized the active site to the Domain III AB section of the AFP molecule. However, a problem with all of these recombinant molecules is their low yield, approximately 100 µg of protein per 3 x 10⁸ SF-9

cells. While we were in the process of making Domain III A, Dr. Mizejewski reported on an active 34-mer peptide (amino acids 447-480) in Domain III B of AFP which inhibited estrogen-stimulated mouse uterine growth (47). We have entered into a collaboration with Dr. Mizejewski and have tested this peptide in our estrogen-dependent uterine growth assay. At this writing we have had variable results with this peptide. In our first experiment there was 37% growth inhibition, which compared favorably with Hep G-2 derived AFP (44% inhibition). In the second experiment there was only 18% growth inhibition, which was not significant in our statistical analysis. One of the problems with the peptide is that it tends to aggregate in solution, which inactivates its function. These microaggregates have been detected by using molecular sieving columns and by NMR spectroscopy. Aggregation increases proportional to the concentration and time of peptide in solution. We are currently working with Dr. Mizejewski, trying to resolve this problem. Dr. Mizejewski has synthesized and tested another peptide (amino acids 511-560) in Domain III C of AFP, and we have synthesized and tested other peptides (amino acids 434-444 and 428-444) in Domain III A of AFP, and none of these peptides has demonstrated activity by inhibiting estrogen-stimulated growth of immature mouse uterus. The two very significant advantages of using a peptide such as AFP 447-480 are that it does not require incubation with lipophilic substances to convert it to its active form and it can be produced in large quantity with substantially less effort required of recombinant systems.

CONCLUSIONS AND FUTURE WORK

Conclusions

1. The site of AFP which is active in blocking estrogen-dependent breast cancer growth is localized in the third domain of the molecule and appears to be contained in a 34-mer amino acid sequence found in domain III B. The functionality of this site is labile, as it tends to aggregate with itself upon standing in solution.
2. Increasing the dose of full-length AFP (10 μ g or above) exempts the protein from the ligand-induced activation requirement and simplifies its use for therapeutics. At this higher dose, full-length natural human AFP stopped the growth of estrogen-dependent breast cancers and androgen-dependent prostate cancer. It did not affect the growth of estrogen-independent breast, ovarian and endometrial cancer and androgen-independent prostate cancer.
3. Positivity for sex steroid hormone receptors appears to be a marker of tumor sensitivity to the growth-inhibitory effects of AFP.
4. Elevations in serum FSH and E_2 appear to be intermediate markers of AFP's ability to interfere with estrogen-dependent responses *in vivo*.
5. The growth-inhibitory effects of AFP are reflected in a histomorphometric profile of cytostasis whereby cells pile up in the G_0G_1 phase of the cell cycle and cell renewal is repressed.

Future Work During Extension Year

1. Stabilize and augment the functionality of the 34-mer peptide (amino acids 447-480 from human AFP) that inhibits estrogen-stimulated mouse uterine growth.
2. Test the effect of full-length natural human AFP against additional estrogen-dependent human breast cancer cell lines and freshly resected patient breast cancers, both grown as xenografts in SCID mice.
3. Complete the development of the assay for AFP receptor and determine the AFP receptor status of the cell lines which were grown as xenografts and already tested for sensitivity to AFP.
4. In mice bearing human breast cancer xenografts, test serum samples before, during and after treatment with AFP for levels of E₂, FSH, LH and CA 27.29.
5. Further evaluate the histomorphometric changes in breast cancer xenografts while they are under the growth-inhibitory influence of AFP.

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PERSONNEL RECEIVING PAY

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